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# Polymerase I and Transcript Release Factor Regulates Lipolysis via a Phosphorylation-Dependent Mechanism

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**OBJECTIVE**—Polymerase I and transcript release factor (PTRF) is a protein highly expressed in adipose tissue and is an integral structural component of caveolae. Here, we report on a novel role of PTRF in lipid mobilization.

**RESEARCH DESIGN AND METHODS**—PTRF expression was examined in different adipose depots of mice during fasting, refeeding, and after administration of catecholamines and insulin. Involvement of PTRF during lipolysis was studied upon PTRF knockdown and overexpression and mutation of PTRF phosphorylation sites in 3T3-L1 adipocytes.

**RESULTS**—PTRF expression in mouse white adipose tissue (WAT) is regulated by nutritional status, increasing during fasting and decreasing to baseline after refeeding. Expression of PTRF also is hormonally regulated because treatment of mice with insulin leads to a decrease in expression, whereas isoproterenol increases expression in WAT. Manipulation of PTRF levels revealed a role of PTRF in lipolysis. Lentiviral-mediated knockdown of PTRF resulted in a marked attenuation of glycerol release in response to isoproterenol. Conversely, overexpressing PTRF enhanced isoproterenol-stimulated glycerol release. Mass-spectrometric analysis revealed that PTRF is phosphorylated at multiple sites in WAT. Mutation of serine 42, threonine 304, or serine 368 to alanine reduced isoproterenol-stimulated glycerol release in 3T3-L1 adipocytes.

**CONCLUSIONS**—Our study is the first direct demonstration for a novel adipose tissue-specific function of PTRF as a mediator of lipolysis and also shows that phosphorylation of PTRF is required for efficient fat mobilization. *Diabetes* 60:757–765, 2011

**W**hite adipose tissue (WAT) plays a critical role in energy homeostasis. Adipocytes store energy in the form of triacylglycerides (TAG) during feeding and breakdown TAG during fasting to release fatty acids and glycerol. TAG breakdown (lipolysis) is regulated by lipolytic hormones, such as catecholamines, whereas lipogenic hormones, such as insulin, stimulate the synthesis of TAG and inhibit lipolysis (1). Catecholamines stimulate lipolysis through increasing cAMP levels and activating protein kinase A (PKA) (2), whereas insulin inhibits lipolysis through activating

phosphodiesterase 3B, which causes degradation of cAMP and loss of PKA activation (3). PKA phosphorylates target proteins, including lipolytic enzymes, and lipid droplet-associated proteins, including hormone-sensitive lipase (HSL) and perilipin (4–6). Phosphorylated HSL translocates from the cytosol to the surface of the lipid droplets where it catalyzes the hydrolysis of TAG together with adipose triglyceride lipase (ATGL) (7).

Polymerase I and transcript release factor (PTRF), also called cav-p60 and cavin, is a 50-kDa protein that is highly expressed in smooth muscle, lung, and adipose tissue. PTRF originally was identified in the nucleus of a mouse embryonic fibroblast cell line, where it plays a role in enhancing the transcriptional activity of polymerase I and polymerase II (8,9). In addition to its nuclear localization, PTRF is found at the cytoplasmic face of caveolae and is the major caveolae-associated protein in primary human adipocytes (10). Recently, PTRF was found to be required for caveolae formation and may serve as a caveolar coat protein together with caveolin-1 (11–13). Of note, it also has been demonstrated that PTRF localizes specifically to a caveolae subclass that metabolizes TAG (14,15), suggesting a potential role of PTRF in TAG metabolism. Moreover, PTRF interacts with HSL in primary human adipocytes and translocates from the plasma membrane to the cytosol in response to insulin treatment, suggesting that PTRF may function in concert with HSL in the regulation of lipolysis (16). Deletion of PTRF causes loss of caveolae and dyslipidemia in mice (17). In humans, mutations in the *PTRF* gene are associated with muscular dystrophy and lipodystrophy (18). These findings point to a potential role of PTRF in regulating lipid metabolism in adipose tissue.

PTRF is phosphorylated at multiple sites (19–23). In primary human adipocytes, phosphorylation of PTRF at multiple serine and threonine residues has been reported (10,14). Insulin induces phosphorylation of PTRF on a tyrosine residue in adipocytes (24,25). However, the functional significance of PTRF phosphorylation has not previously been characterized.

Here, we report that PTRF expression is under nutritional and hormonal control in the WAT of mice. Fasting and catecholamine treatment induce PTRF expression, whereas refeeding and insulin administration reduce its expression in WAT. Lentiviral-mediated reduction of PTRF expression in 3T3-L1 adipocytes significantly decreases isoproterenol-stimulated glycerol release. Conversely, overexpression of PTRF leads to increased isoproterenol-stimulated glycerol release. Changes in PTRF expression are accompanied by phosphorylation because PTRF is phosphorylated during fasting and upon isoproterenol treatment in adipocytes, and this phosphorylation is PKA dependent. Mutation of serine 42, threonine 304, or serine 368 to alanine of PTRF leads to reduced isoproterenol-stimulated

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glycerol release. Taken together, these data suggest that PTRF plays a crucial role in lipolysis and that this function is dependent on phosphorylation.

## RESEARCH DESIGN AND METHODS

All procedures were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee. Male C57BL/6 mice, aged 18–20 weeks, were maintained in a temperature-controlled environment at 24 °C under a 12-h light/12-h dark cycle (0600–1800 h) with ad libitum access to a standard diet (F6 rodent diet; Harlan Teklad, Madison, WI) and water or water alone when fasted. Mice were killed between 0900 and 1100 h and rapidly dissected. Tissues were flash-frozen for further analysis.

**Cell culture and differentiation.** 3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) with 10% bovine calf serum at 5% CO<sub>2</sub>. Two days after confluence was reached, cells were differentiated by using 1  $\mu$ M dexamethasone (Sigma-Aldrich, St. Louis, MO), 10  $\mu$ g/mL insulin (Invitrogen), and 0.5 mmol/L isobutylmethylxanthine (Sigma-Aldrich) in DMEM with 10% FBS and then maintained in medium containing insulin for another 2 days. Cells were then maintained in DMEM with 10% FBS until they were ready to be harvested. For insulin and isoproterenol experiments, adipocytes were incubated in serum-free DMEM containing 2% BSA (Sigma-Aldrich) for 16 h then treated with insulin or isoproterenol for the times and at the concentrations indicated.

**Overexpression, site-directed mutagenesis, and short-hairpin RNA-mediated knockdown of PTRF.** For overexpression of PTRF, full-length PTRF cDNA were subcloned into a lentiviral expression vector pCDH-CMV-EF1-Puro (System Biosciences, Mountain View, CA). Mutation of PTRF phosphorylation sites to alanine was generated using the QuickChange II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) and PTRF-pCDH-CMV-EF1-Puro as a template according to the manufacturer's instructions. For PTRF knockdown, five PTRF short-hairpin RNA (shRNA) constructs in lentiviral vector pLKO.1 were obtained from Open Biosystems (Huntsville, AL). The construct chosen for the knockdown experiments was TRCN0000103575. All lentiviral constructs were transfected into 293T cells using Lipofectamine 2000 (Invitrogen), along with packaging and envelope plasmids expressing gag, pol, rev, and VSV-G genes, respectively. Supernatants were collected after 48 h. After filtration to remove cell debris, supernatants were added to 3T3-L1 adipocytes at day 5 after differentiation, and cells were studied 5 days after infection.

**RNA extraction and gene expression analysis.** Total RNA was extracted from cell lysates or homogenized tissues using an RNeasy Lipid Tissue kit for PCR (Qiagen, Germantown, MD), followed by generation of cDNA using a QuantiTect Reverse Transcription kit (Qiagen, Germantown, MD). Quantitative real-time PCR was performed using the 7800HT (Applied Biosystems, Foster City, CA) thermal cycler and SYBR-Green master mix (Applied Biosystems). Primers used were PTRF forward 5'-AGTGAGCTCAAAGCCAGCAT-3', reverse 5'-GCCTTAGTCCCCCAAAGAC-3', and 36B4 forward 5'-AGATTCGGGATATGCTGTGGC-3', reverse 5'-TCGGGTCTAGACAGTGTTC-3'. Relative expression levels were calculated by the standard-curve method.

**SDS-PAGE and immunoblotting.** Proteins were extracted from adipose tissue and 3T3-L1 cells in a radioimmunoprecipitation assay buffer containing 0.5 mmol/L Tris-HCl, pH 7.4; 150 mmol/L NaCl; 1 mmol/L EDTA; 1% NP40; 0.5% sodium deoxycholate; 2 mmol/L sodium orthovanadate; 1 mmol/L sodium fluoride; 1 mmol/L sodium pyrophosphate; 2.5 mmol/L  $\beta$ -glycerolphosphate; and protease inhibitors (Roche, Indianapolis, IN). Extracts were subjected to SDS-PAGE (10.5–14% gels; Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose membranes (Millipore, Bedford, MA). After blocking, membranes were incubated with mouse anti-PTRF (BD Transduction Laboratories, San Jose, CA), guinea pig anti-perilipin, rabbit anti-HSL (Cell Signaling Technology, Danvers, MA), rabbit anti-phospho-HSL (serine 660) (Cell Signaling Technology), rabbit anti-caveolin-1 (BD Transduction Laboratories), mouse anti-RAN (BD Transduction Laboratories), or mouse anti- $\beta$ -actin (Abcam, Cambridge, MA). Antibody binding was detected using secondary antibodies conjugated to horseradish peroxidase, followed by chemiluminescence detection with SuperSignal West Pico Chemiluminescence Substrate (Pierce, Rockford, IL).

**Alkaline phosphatase treatment.** Protein extracts from WAT were treated with alkaline phosphatase (1 unit/ $\mu$ g protein) (New England Biolabs, Ipswich, MA) for 1 h at 37°C.

**Immunoprecipitation and liquid chromatography/tandem mass spectrometry.** Lysates were immunoprecipitated using mouse anti-PTRF antibodies and collected using protein A/G PLUS-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitated proteins were solubilized directly into Laemmli sample buffer. For all mass spectrometry (MS) experiments, PTRF immunoprecipitates were separated using SDS-PAGE, and a PTRF band was excised from Coomassie blue-stained gel. Samples were

subjected to reduction with dithiothreitol, alkylation with iodoacetamide, and in-gel digestion with trypsin overnight at pH 8.3, followed by liquid chromatography/tandem MS (LC/MS/MS) performed using EASY-nLC nano-flow high-performance liquid chromatography (Proxeon Biosciences, Odense, Denmark) with a self-packed 75- $\mu$ m id  $\times$  15 cm C<sub>18</sub> column connected to a hybrid LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, Waltham, MA) in the data-dependent acquisition and positive-ion mode at 300 nL/min. MS/MS spectra collected via collision-induced dissociation in the ion trap were searched against the concatenated target and decoy (reversed) single-entry PTRF and full Swiss-Prot protein databases using Sequest (Proteomics Browser Software, Thermo Scientific) with differential modifications for serine/threonine/tyrosine phosphorylation (+79.97). Phosphopeptide sequences were identified if they initially passed the following Sequest scoring thresholds against the target database: 1 + ions, Xcorr  $\geq$  2.0, Sf  $\geq$  0.4,  $P \geq$  5; 2 + ions, Xcorr  $\geq$  2.0, Sf  $\geq$  0.4,  $P \geq$  5; and 3 + ions, Xcorr  $\geq$  2.60, Sf  $\geq$  0.4,  $P \geq$  5 against the target protein database. Passing MS/MS spectra were manually inspected to be sure that all b- and y-fragment ions aligned with the assigned sequence and modification sites. Determination of the exact sites of phosphorylation was aided using FuzzyLons and GraphMod, and phosphorylation site maps were created using ProteinReport software (Proteomics Browser Software Suite, Thermo Scientific). False-discovery rates of peptide hits were estimated below 1.25% based on reversed database hits.

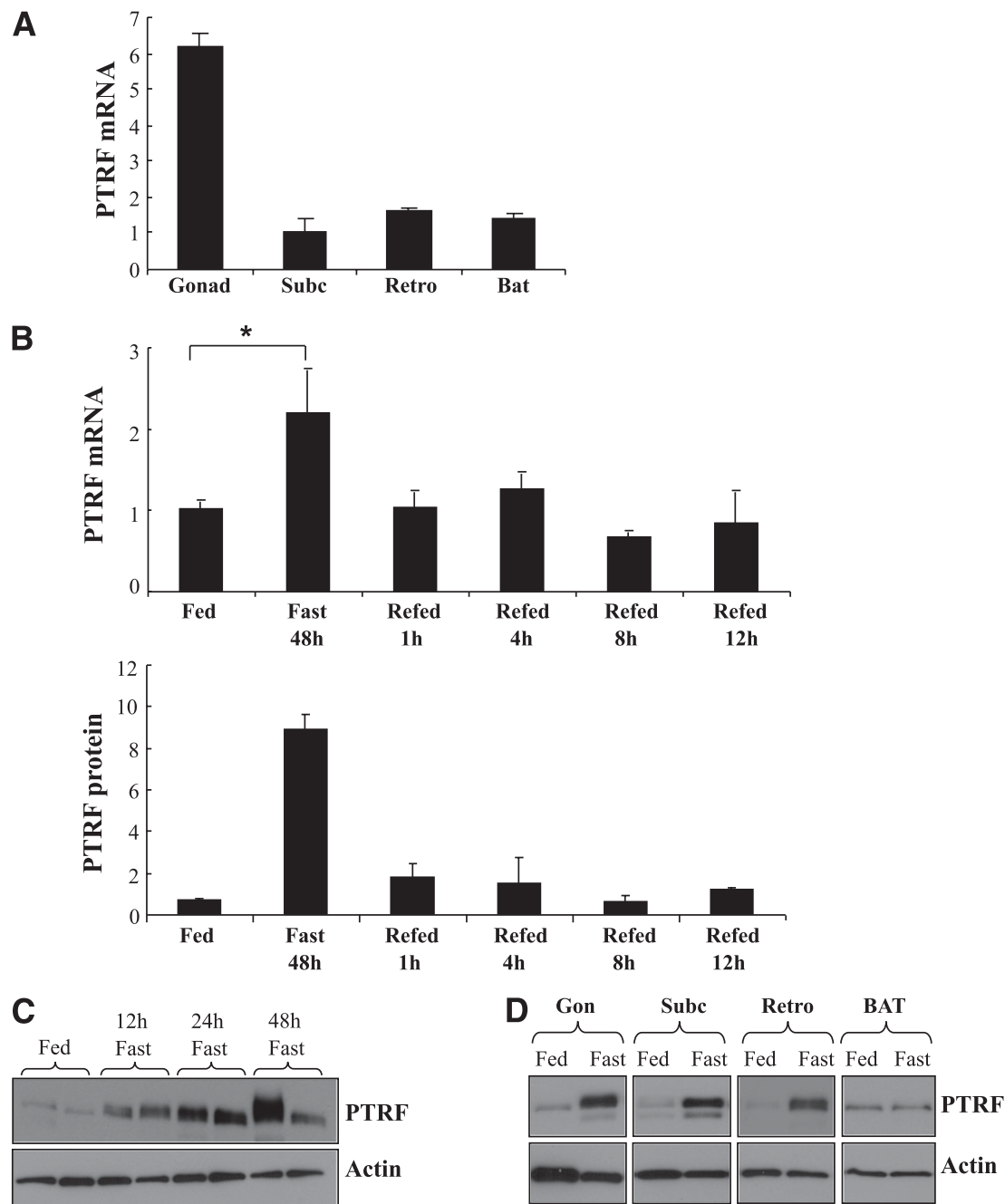
**Lipolysis assays.** For basal and stimulated lipolysis, adipocytes were serum starved overnight and incubated in the presence or absence of isoproterenol for the indicated time. Glycerol release was measured using the free glycerol reagent kit (Sigma). Adipocytes were harvested in radioimmunoprecipitation assay buffer containing protease inhibitors, and the protein content of cell lysates was determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Glycerol levels were normalized with total cellular protein.

## RESULTS

### PTRF expression in adipose tissue is regulated by nutritional status in mice in a depot-specific manner.

PTRF is highly expressed in adipose tissue (10). We examined its expression levels in different adipose depots in mice. PTRF expression was depot specific, with the highest expression levels in gonadal adipose tissue compared with subcutaneous, retroperitoneal, and brown adipose tissue depots (Fig. 1A). Of note, no significant difference in PTRF protein expression was seen in any of the adipose tissue depots of *ob/ob* mice compared with wild-type mice (Supplementary Fig. 2). We next examined nutritional influences on PTRF expression. In gonadal fat, expression of PTRF was regulated by fasting and refeeding. Fasting for 48 h leads to a twofold induction in mRNA levels and a ninefold increase in protein levels compared with fed mice (Fig. 1B). The magnitude of PTRF mRNA changes was lower than that seen in protein, suggesting that PTRF expression is also modulated on the posttranscriptional level. Refeeding resulted in a rapid reduction of PTRF expression to fed levels within 1 h and remained at the same levels throughout the refeeding time course (Fig. 1B). The observed increase in PTRF expression in gonadal adipose tissue was time dependent (Fig. 1C). Furthermore, PTRF expression responds to nutritional manipulation in all three WAT depots examined but not in brown adipose tissue. Although PTRF expression levels increased after a 24-h fast in WAT depots, including gonadal, subcutaneous, and retroperitoneal, expression in brown adipose tissue did not change upon fasting (Fig. 1D). A faster-migrating PTRF band was observed (Fig. 1D), which is a result of well-characterized proteolytic cleavages of PTRF in adipocytes (10). We also observed a slower-migrating PTRF band on SDS-PAGE in WAT upon fasting (Fig. 1C and D), suggesting a posttranslational modification of PTRF, such as phosphorylation.

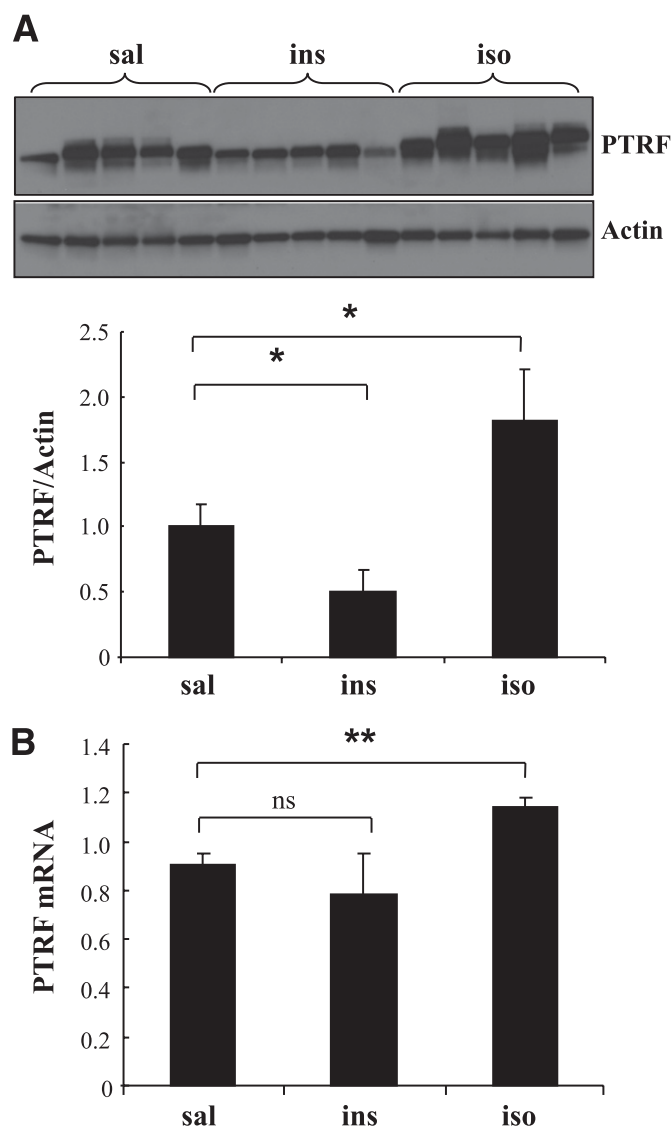
**PTRF expression is regulated by insulin and catecholamines in WAT.** Insulin and catecholamines play a role in the response of adipose tissue to fasting and refeeding. We therefore evaluated the direct effects of



**FIG. 1.** PTRF expression is upregulated in WAT but not in brown adipose tissue upon fasting in mice. **A:** PTRF mRNA expression in gonadal (Gonad), subcutaneous (Subc), retroperitoneal (Retro), and brown adipose tissue (Bat). **B:** PTRF mRNA (*upper panel*) and protein (*bottom panel*) expression in gonadal adipose tissue from ad libitum-fed or fasted mice for 48 h or refeed mice for the indicated time. **C:** PTRF expression levels were determined by immunoblot in gonadal adipose tissue of ad libitum-fed or fasted mice for 12, 24, and 48 h. **D:** PTRF protein expression in gonadal (Gonad), subcutaneous (Subc), retroperitoneal (Retro), and brown adipose tissue (Bat) of ad libitum-fed or fasted mice for 24 h. Gene expression and protein expression were normalized to 36B4 and to actin, respectively ( $n = 4-8$ ). Data are expressed as means  $\pm$  SE. \* $P < 0.05$ .

these hormones on PTRF expression in vivo. Mice were injected intraperitoneally with insulin (0.75 units/kg) or isoproterenol (10 mg/kg), and the expression levels of PTRF were measured 1-h postinjection. Insulin treatment resulted in a twofold reduction of PTRF protein levels compared with that in the WAT of saline-treated control mice (Fig. 2A). Conversely, treatment with isoproterenol resulted in a twofold increase in PTRF protein levels in adipose tissue compared with controls (Fig. 2A). Isoproterenol treatment also induced a slower-migrating

PTRF band on SDS-PAGE (Fig. 2A) similar to that seen in response to fasting (Fig. 1C and D), suggesting that PTRF might undergo phosphorylation. The magnitude of PTRF mRNA changes in response to hormonal treatment was lower than changes in protein expression (Fig. 2B), again suggesting a posttranscriptional regulation of PTRF. Taken together, these results indicate that PTRF expression in WAT is under nutritional regulation and that this tight regulation might be mediated directly by insulin and catecholamine, suggesting a role of PTRF in lipolysis.



**FIG. 2.** PTRF expression is regulated by insulin and catecholamine in gonadal adipose tissue. PTRF expression in gonadal adipose tissue of mice fasted for 4 h and intraperitoneal injected with either saline (sal), insulin (ins) (0.75 units/kg), or isoproterenol (iso) (10 mg/kg). Tissues were harvest 1 h after injection. **A:** Immunoblot using PTRF antibodies (upper panel). PTRF protein levels normalized to actin levels (bottom panel). **B:** PTRF gene expression normalized to 36B4 expression ( $n = 5$ ). Data are expressed as means  $\pm$  SE. \* $P < 0.05$ ; \*\* $P < 0.01$ . ns, not significant.

### Identification of PTRF phosphorylation sites in WAT.

We observed a slower-migrating PTRF band in WAT upon fasting (Fig. 1C and D). This was reversed by alkaline phosphatase treatment, suggesting that this shift in mobility is secondary to phosphorylation (Supplementary Fig. 1). Using LC/MS/MS, we then identified the phosphorylation sites of PTRF in the WAT of mice fasted for 24 h. Table 1 summarizes the identified phosphorylation sites of PTRF. Four of the identified phosphorylation sites of PTRF, namely serine 169, serine 171, threonine 304, and serine 368, were predicted to be phosphorylated by PKA when analyzed by NetPhosK software (26), which is consistent with the increased activity of PKA during fasting (27). These data suggest a possible role of PTRF phosphorylation during TAG mobilization during fasting.

**PTRF expression and phosphorylation is hormonally regulated in 3T3-L1 adipocytes.** Because 3T3-L1 adipocytes provide a cell culture model system for studies of lipolysis regulation, we used these cells to assess the role of PTRF and its phosphorylation in lipolysis. We first confirmed the *in vivo* data on PTRF regulation and phosphorylation. Consistent with the nutritional and hormonal regulation of PTRF expression *in vivo* in WAT, PTRF expression *in vitro* in 3T3-L1 adipocytes also is regulated by serum depletion, insulin, and catecholamine. Serum depletion of 3T3-L1 adipocytes for 16 h resulted in a twofold upregulation of PTRF protein levels (Fig. 3A, left panel). The addition of insulin to serum-free media resulted in a decrease of PTRF protein levels (Fig. 3A, right panel) in a dose-dependent manner (Fig. 3B). PTRF expression decreased by 50% compared with control levels (Fig. 3B). At a concentration of 100 nmol/L insulin, reduction of PTRF levels was first seen after 2 h and persisted throughout the 24-h time course (Fig. 3C).

In contrast to insulin, isoproterenol treatment increased PTRF protein levels with a peak at 1–2 h after treatment of 3T3-L1 adipocytes (Fig. 3D). PTRF levels remained elevated from baseline up to 8 h after isoproterenol treatment and decreased to basal levels between 8 and 24 h (data not shown). PTRF also was phosphorylated in 3T3-L1 adipocytes in response to isoproterenol treatment, as indicated by the appearance of a slower-migrating PTRF band, which first appears at 0.1  $\mu$ mol/L isoproterenol (Fig. 3E) and within 30 min after the incubation of 3T3-L1 adipocytes with 1  $\mu$ mol/L isoproterenol (Fig. 3D). This slower-migrating band disappeared almost completely 240 min after isoproterenol incubation (Fig. 3D). This is in agreement with the phosphorylation of PTRF in WAT in response to fasting and isoproterenol treatment (Figs. 1 and 2).

Because isoproterenol induces lipolysis through activating PKA-dependent phosphorylation of lipolytic proteins, including HSL and perilipin (1), we examined the role of PKA on PTRF phosphorylation. Incubation of 3T3-L1 adipocytes with isoproterenol in the presence and absence of H89, a PKA inhibitor, substantially inhibited isoproterenol-induced phosphorylation of PTRF (Fig. 3F). Perilipin also migrates more slowly during SDS-PAGE upon isoproterenol treatment as a result of phosphorylation by PKA on multiple sites (6). As expected, treatment with H89 also reduced the PKA-induced phosphorylation of perilipin (Fig. 3F). This indicates that PTRF undergoes PKA-dependent phosphorylation upon isoproterenol treatment of 3T3-L1 adipocytes, in agreement with our mass spectrometric identification of PKA-phosphorylation sites in PTRF in WAT during fasting (Table 1).

Taken together, these *in vitro* results confirm the hormonal-regulated expression and phosphorylation of PTRF seen in WAT.

**Loss and gain of function of PTRF have opposite effects on lipolytic activity in 3T3-L1 adipocytes.** The regulation of PTRF expression by insulin and catecholamines in adipocytes suggested a possible role for PTRF in lipolysis. To assess such a potential role for PTRF, we suppressed its expression by using lentiviral-driven shRNA targeting PTRF in 3T3-L1 adipocytes. PTRF protein levels were decreased by 45% compared with adipocytes infected with the nontargeting control shRNA lentivirus (Shcont) (Fig. 4A). Although basal rates of lipolysis were not affected by PTRF knockdown, isoproterenol-stimulated lipolysis, assayed by glycerol release, revealed a 30–40% reduction following PTRF knockdown (Fig. 4B).

TABLE 1

Identification of PTRF phosphorylation sites in WAT by LC/MS/MS experimental and actual peptide sequence

Peptide sequence	Site	Sequest final score	MH+	Difference in mass (ppm)	Sequest cross-correlation score	$\Delta C_n$	Sequest preliminary score
PYSGFPDASsEGPEPTQGEAR	Ser21	0.67	2258.9	-0.3	4.5	0.05	404
ATEEPSGTGsDELK	Ser42	0.87	1613.7	-3.1	4.03	0.07	1131
LPAKLsVSK	Ser169	0.88	1022.6	0.4	3.34	0.22	390
VMIYQDEVKLPAKLSVsK	Ser171	0.40	2128.1	-7.1	3.48	0.08	151
SFTPDHVYAR	Thr304	0.59	1371.6	1.1	2.34	0.19	684
GSSPDVHTLLEITEESDAVLVDK	Ser368	0.73	2534.2	2.6	4.69	0.17	486

PTRF was immunoprecipitated from the gonadal adipose tissue of mice fasted for 24 h. Peptides obtained from in-gel tryptic digestion of the PTRF band were subjected to LC/MS/MS. Identified PTRF phosphopeptide sequences are listed below, along with the position of the phosphorylation site in the PTRF sequence and relevant database search scores. Lowercase boldface s and t indicate phosphorylation at serine and threonine, respectively. Phosphorylation at Ser42, Ser169, and Ser368 were previously identified, whereas Ser21, Ser171, and Thr304 are novel PTRF phosphorylation sites.  $\Delta C_n$ , Sequest cross-correlation score difference between the top-ranked and next-best peptide sequence.

Next, we overexpressed PTRF in 3T3-L1 adipocytes by infecting cells with lentivirus containing full-length PTRF cDNA. PTRF protein levels increased twofold compared with adipocytes infected with green fluorescent protein (GFP) control lentivirus (Fig. 4C). This was associated with a 25–35% increase in isoproterenol-stimulated glycerol release (Fig. 4D). PTRF overexpression had no effects on the basal rate of lipolysis (Fig. 4D). These results are consistent with a role for PTRF during lipolysis.

**Mutation of specific PTRF phosphorylation sites reduces lipolysis in 3T3-L1 adipocytes.** We next examined the effects of PTRF phosphorylation on lipolysis. We generated lentiviral constructs containing GFP as control, wild-type PTRF, or PTRF mutants in which PTRF phosphorylation sites (Table 1) were mutated to alanine. 3T3-L1 adipocytes were infected with lentivirus, and the effects of PTRF mutations were examined 5 days after infection. Isoproterenol-stimulated lipolytic activity, as assayed by glycerol release, was lower in adipocytes expressing PTRF mutants S42A, T304A, and, S368A, when compared with wild-type PTRF (Fig. 5A). On the other hand, no effect was seen in the PTRF mutants S21A, S169A, and S171A, because they all showed similar lipolytic activity as wild-type PTRF (Fig. 5A and data not shown). There was no significant effect of PTRF phosphorylation-site mutations on basal lipolysis (Fig. 5A and Supplementary Table 1). These results suggest that PTRF regulation of isoproterenol-stimulated lipolysis is dependent on serine and threonine phosphorylation of PTRF at Ser42, Thr304, or Ser368 residues.

It is known that HSL, the key enzyme responsible for lipolysis in WAT, interacts with PTRF (16) and that its function during lipolysis also is dependent on PKA phosphorylation (28). To gain insight into the mechanisms by which the mutation of PTRF phosphorylation sites suppresses lipolysis, we examined the phosphorylation status of HSL in these cells. Notably, we found that the suppressed lipolytic activity in adipocytes expressing the PTRF mutations S42A, T304A, or S368A (Fig. 5A) was accompanied by a reduction of HSL phosphorylation at serine 563 and serine 660 (Fig. 5B). PKA-mediated phosphorylation of perilipin, on the other hand, was not affected in cells expressing mutated phosphorylation sites of PTRF (data not shown). In addition, there was no effect in the PTRF mutants on HSL phosphorylation at serine 565 (data not shown), which has been shown to be a target for

AMP kinase but not PKA (28). Taken together, these data indicate that phosphorylation of PTRF at serine 42, threonine 304, or serine 368 is essential for the activation of HSL by PKA during lipolysis.

## DISCUSSION

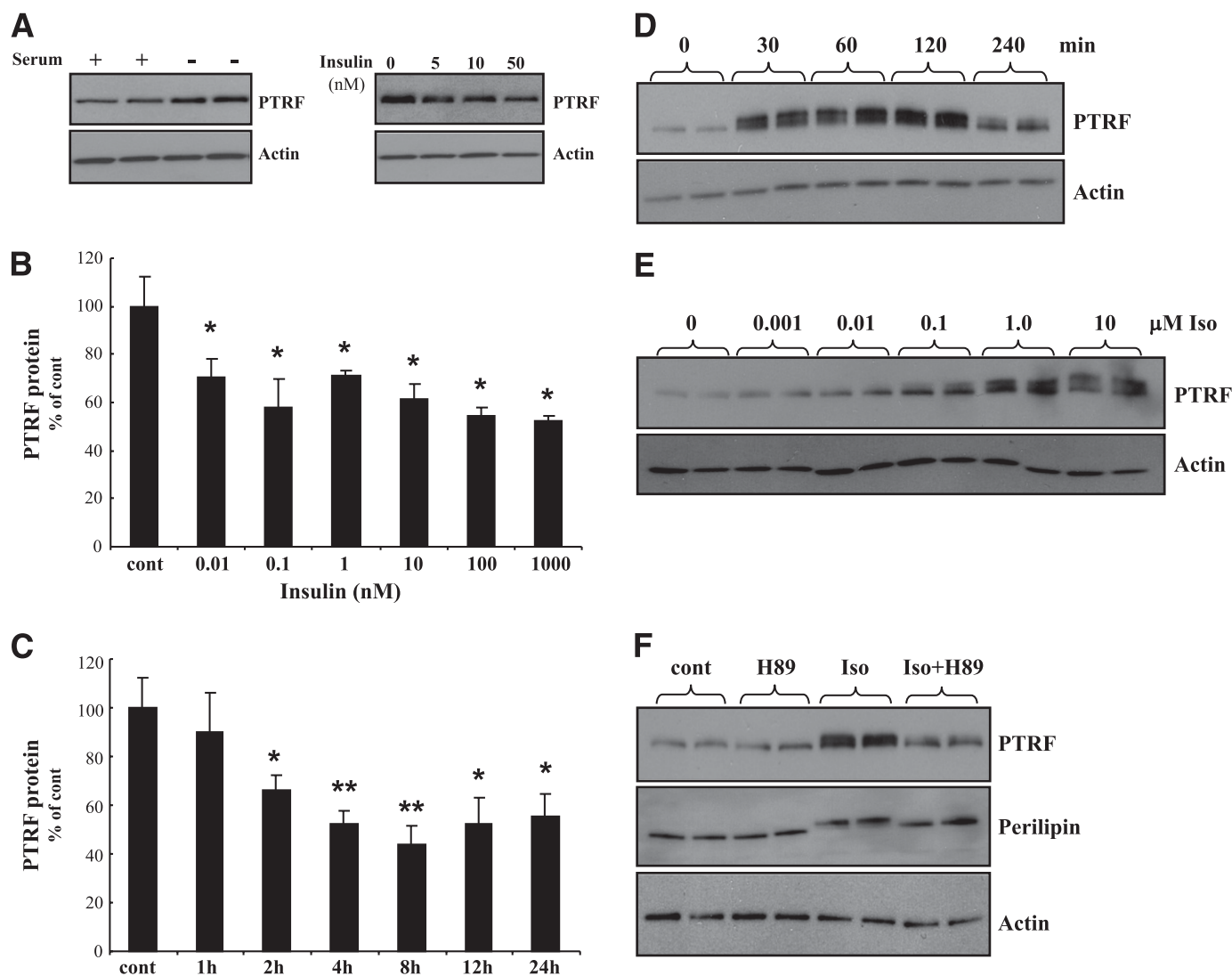
Adipose tissue lipolysis is an important process in which TAG are mobilized, releasing fatty acids and glycerol and thus providing the body with substrates during fasting. The dysregulation of lipolysis, which can occur in obesity or lipodystrophic states, can lead to elevated levels of fatty acids in the circulation, which is associated with an increased risk for developing cardiovascular diseases and diabetes. The lipolytic machinery is complex and includes as central components perilipin, HSL, and ATGL, in addition to several factors and interacting proteins. Here, we present data suggesting PTRF as a novel physiologically regulated and critical component of the lipolytic machinery.

We found that levels of PTRF expression vary in different adipose tissue depots. Moreover, PTRF expression is under strict hormonal and nutritional control in WAT but not in brown adipose tissue. Although catecholamines and fasting increase PTRF expression, insulin and refeeding decrease its expression. This differential regional expression and regulation of PTRF might contribute to the metabolic heterogeneity observed among different adipose depots (29,30). The induction of PTRF expression by fasting and catecholamines in WAT supports with a role of PTRF during lipid mobilization. Of note, PTRF also is expressed in skeletal muscle, another tissue depot that exhibits lipolytic activity. Future experiments will determine whether skeletal muscle PTRF also could be regulated in a similar manner.

Consistent with nutritional and hormonal regulation, manipulation of PTRF expression in cultured cells also changed lipid homeostasis. PTRF overexpression and knockdown resulted in enhancement and suppression of lipolysis, respectively. This is in line with PTRF subcellular location at the lipid droplets and at TAG-synthesizing caveolae subclass.

Lipolysis is an important process in which TAG are mobilized, releasing fatty acids and glycerol and thus providing the body with substrates during fasting. The lipolytic machinery is complex and includes as central components



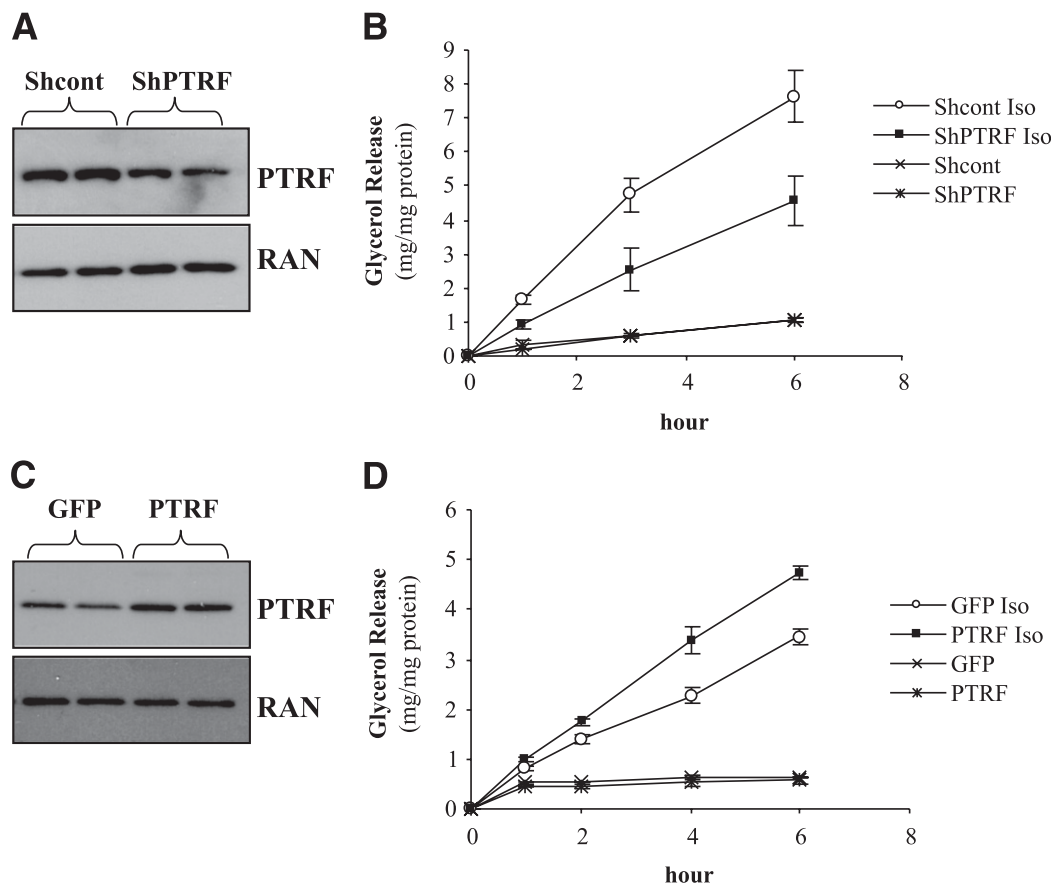


**FIG. 3.** PTRF expression is regulated by insulin and catecholamine in 3T3-L1 adipocytes. **A:** PTRF immunoblot of protein extracts from fully differentiated 3T3-L1 adipocytes and incubated for 16 h in either 10% FBS (+) or serum-free (-) DMEM (*left panel*) or serum-free DMEM supplemented with insulin at the concentrations indicated (*right panel*). **B:** Dose-response curve for PTRF protein expression were generated by incubating 3T3-L1 adipocytes in serum-free DMEM supplemented with insulin at the indicated concentrations for 16 h (three experiments,  $n = 3$ ). **C:** Time course for PTRF protein expression were generated by incubating 3T3-L1 adipocytes in serum-free DMEM supplemented with 100 nmol/L insulin for the indicated time. Data were normalized to actin and expressed relative to control cells. **D:** Time course for PTRF protein expression was generated by incubating 3T3-L1 adipocytes in serum-free DMEM supplemented with 1  $\mu$ mol/L isoproterenol for the indicated time. **E:** Dose-response curves for PTRF protein expression were generated by incubating 3T3-L1 adipocytes in serum-free DMEM supplemented with isoproterenol at the indicated concentrations for 3 h (three experiments,  $n = 3$ ). **F:** 3T3-L1 adipocytes were preincubated with the PKA inhibitor H89 (100  $\mu$ mol/L) for 1 h prior to treatment with 1  $\mu$ mol/L isoproterenol (Iso) for 1 h. Phosphorylation of PTRF is indicated by the appearance of an additional PTRF band with slower migration in SDS-PAGE. Immunoblots were performed with the indicated antibody. Actin immunoblot was used as a loading control. Data are expressed as means  $\pm$  SE. \* $P < 0.05$ ; \*\* $P < 0.01$ .

perilipin, HSL, and ATGL in addition to several factors and interacting proteins. Recently, both HSL and perilipin were localized to TAG-synthesizing caveolae subclass (31). Other lipolytic components, such as the  $\beta$ -adrenergic receptors, as well as PKA also were found to associate with caveolar membranes (32). It is therefore possible that PTRF, together with other lipolytic proteins, facilitates lipolysis of TAG in lipid droplets as well as in caveolae. This is consistent with the finding that the localization of both PTRF and HSL in caveolae is under insulin control, where insulin, which inhibits lipolysis, induced translocation of both PTRF and HSL from caveolae to the cytosol (16).

During fasting, hormones including catecholamines induce lipolysis through binding to  $\beta$ -adrenergic receptors, activating PKA, which phosphorylates perilipin and HSL

at multiple sites (5,28,33,34). PKA-dependent phosphorylation of HSL is necessary for docking of HSL at the surface of lipid droplets and for activation of lipolysis (28). However, localization of PTRF to caveolae in the plasma membrane is not influenced by  $\beta$ -adrenergic stimulation (16). Insulin, on the other hand, inhibits lipolysis through activating phosphodiesterase 3B, which causes degradation of cAMP and loss of PKA activation (3). Our data show that during lipolysis, PTRF also is phosphorylated by PKA at multiple sites and that this PKA-dependent phosphorylation of PTRF plays an essential role during lipolysis. A number of phosphorylation sites in the mouse and human PTRF sequences have been previously identified. However, the functional significance of PTRF phosphorylation has not previously been characterized. Here, we



**FIG. 4.** Effects of PTRF knockdown and overexpression on lipolytic activity in 3T3-L1 adipocytes. PTRF expression was stably suppressed in 3T3-L1 adipocytes by lentiviral-driven shRNA targeting PTRF (ShPTRF). Nontargeting control ShRNA (Shcont) was used as a control. **A:** PTRF protein levels after knockdown were determined by immunoblot with PTRF antibodies. **B:** Basal and isoproterenol-stimulated (10  $\mu$ mol/L) (Iso) glycerol release were evaluated 5 days after infection with lentivirus. Glycerol release was normalized to the total cellular protein. **C:** PTRF was stably overexpressed in 3T3-L1 adipocytes by a lentiviral vector containing full-length PTRF cDNA. Control cells were infected with GFP lentiviral expression vector. Levels of PTRF were determined by immunoblotting. **D:** Basal and isoproterenol-stimulated (10  $\mu$ mol/L) glycerol release were evaluated 5 days after infection with lentivirus. Glycerol release was normalized to the total cellular protein (three experiments,  $n = 4$  per time point). RAN immunoblot was used as a loading control. Data are expressed as means  $\pm$  SE.

provide evidence for an adipocyte-specific functional role of PTRF phosphorylation, as mutation of PTRF at serine 42, threonine 304, or serine 368 to alanine significantly abrogated the lipolytic response in 3T3-L1 adipocytes.

In addition to phosphorylation, the action of HSL is dependent on its interaction with other proteins such as perilipin. This interaction is necessary for translocation of HSL from the cytosol to the lipid droplets during lipolysis (35). Furthermore, HSL interacts with the protein lipotransin, which docks HSL at the surface of the lipid droplets (36). Because PTRF has been shown to interact with HSL (16), it is possible that PTRF also serves as a bridge between HSL and PKA to mediate phosphorylation and activation of HSL during lipolysis. Indeed, the decrease in lipolysis in cells expressing the PTRF mutants S42A, T304A, and S368A was accompanied by a reduction in serine phosphorylation of HSL. Thus, phosphorylation of PTRF is required for the subsequent phosphorylation of HSL and initiation of lipolysis. It remains to be determined whether the interaction between PTRF and HSL is phosphorylation dependent and whether PKA-phosphorylated PTRF interacts with other lipolytic proteins. It also would be of interest to determine whether PTRF itself had TAG hydrolase activity or if it serves specifically to modulate the activity of other hydrolases such as HSL.

Recent studies (11,12,17) have demonstrated that PTRF is required for the formation of caveolae because knockdown of PTRF leads to loss of caveolae. Loss of caveolae was accompanied with rapid degradation of caveolin-1 protein (11). It is unlikely that caveolae biogenesis is affected in cells expressing mutated phosphorylation sites of PTRF because no effects were seen on caveolin-1 protein expression in these cells (data not shown). However, it would be interesting to determine whether PTRF phosphorylation has an effect on caveolae morphology and dynamics.

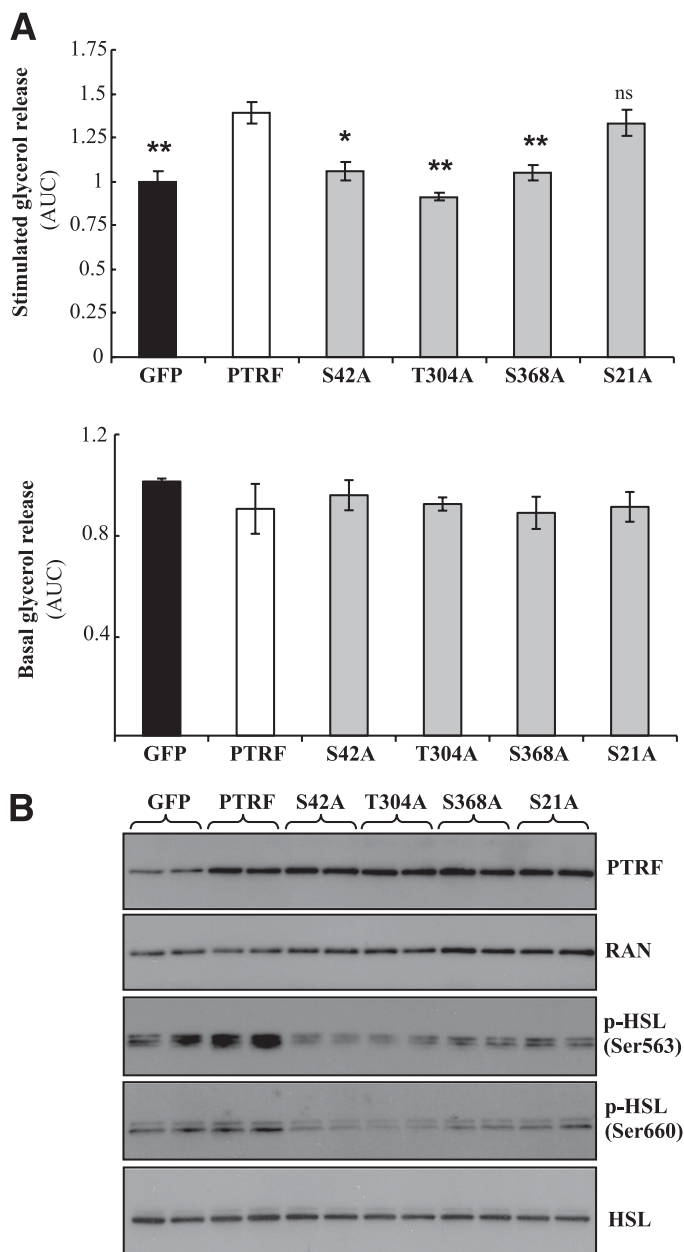
Our study represents the first direct demonstration of nutritional and hormonal control of PTRF expression and phosphorylation in adipose tissue in mouse. We provide evidence for a novel adipose tissue-specific function of PTRF as a critical mediator of lipolysis, which is a central function of the adipocytes.

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**FIG. 5.** Effects of PTRF phosphorylation-site mutations on lipolytic activity in 3T3-L1 adipocytes. 3T3-L1 adipocytes on day 5 of differentiation were infected with lentivirus vector containing, GFP, wild-type PTRF, or PTRF with mutations at serine and threonine phosphorylation sites to alanine (S42A, T304A, S368A, and S21A). **A:** 3T3-L1 adipocytes were treated 5 days postinfection with either 10  $\mu$ M isoproterenol (representing stimulated glycerol release) or vehicle (representing basal glycerol release), and glycerol release was measured at 1, 3, and 6 h after isoproterenol treatment. Glycerol levels were normalized with total cellular protein. Area under the curves were calculated and expressed relative to GFP control levels (three experiments,  $n = 4$  per time point). **B:** Levels of PTRF, HSL, and phosphorylated HSL at serine 563 and 660 (p-HSL [Ser660], Ser563) were determined in adipocytes after isoproterenol (10  $\mu$ M/L) treatment for 2 h by immunoblot with PTRF, HSL, and p-HSL (ser563 and ser660) antibodies. RAN immunoblot was used as a loading control. Data are expressed as means  $\pm$  SE. \* $P < 0.05$ ; \*\* $P < 0.01$ . ns, not significant.

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